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Plant Growth Substances Produced by *Azospirillum brasilense* and Their Effect on the Growth of Pearl Millet (*Pennisetum americanum* L.)†

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Azospirillum brasilense, a nitrogen-fixing bacterium found in the rhizosphere of various grass species, was investigated to establish the effect on plant growth of growth substances produced by the bacteria. Thin-layer chromatography, high-pressure liquid chromatography, and bioassay were used to separate and identify plant growth substances produced by the bacteria in liquid culture. Indole acetic acid and indole lactic acid were produced by *A. brasilense* from tryptophan. Indole acetic acid production increased with increasing tryptophan concentration from 1 to 100 μg/ml. Indole acetic acid concentration also increased with the age of the culture until bacteria reached the stationary phase. Shaking favored the production of indole acetic acid, especially in a medium containing nitrogen. A small but biologically significant amount of gibberellin was detected in the culture medium. Also at least three cytokinin-like substances, equivalent to about 0.001 μg of kinetin per ml, were present. The morphology of pearl millet roots changed when plants in solution culture were inoculated. The number of lateral roots was increased, and all lateral roots were densely covered with root hairs. Experiments with pure plant hormones showed that gibberellin causes increased production of lateral roots. Cytokinin stimulated root hair formation, but reduced lateral root production and elongation of the main root. Combinations of indole acetic acid, gibberellin, and kinetin produced changes in root morphology of pearl millet similar to those produced by inoculation with *A. brasilense*.

There is firm evidence that indole-3-acetic acid (IAA) (1, 3, 5, 7, 17, 29), gibberellins (1, 5, 15, 17), and cytokinins (12, 14, 16, 24, 25, 26), all produced by plants and essential to their growth and development, are produced also by various bacteria which live in association with plants. There is also evidence that the growth hormones produced by the bacteria can in some instances increase growth rates and improve yields of the host plants (1, 4).

It is possible that rhizosphere bacteria capable of fixing nitrogen may improve plant productivity both by hormonal stimulation and by supplying nitrogen. Although many experiments have shown modest improvements in plant growth resulting from addition of bacterial cultures to the rhizosphere, the stimulus involved usually is not identified. This is the case in studies with *Azospirillum* spp., a widespread group of nitrogen-fixing organisms which has received considerable attention in recent years (20).

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Inoculations with *Azospirillum* spp. have increased dry weight yields of plants in both greenhouse (M. H. Gaskins and D. H. Hubbell, Proc. Soil-Root Interface Symp., in press) and field experiments (30). Some investigations have suggested that dinitrogen fixed by the bacteria and subsequently used by the plants is responsible for the accelerated plant growth (8). However, despite extensive research efforts, no one has yet demonstrated conclusively that observed yield increases result from nitrogen fixation by the inoculated organism.

In the experiments reported here we studied phytohormone production in this putative nitrogen-fixing grass-bacteria association to determine whether the bacteria might enhance plant growth by this mechanism.

MATERIALS AND METHODS

Culture. *Azospirillum brasilense* Sp13t SR2 (formerly *Spirillum lipoferum*) is a double-marked, antibiotic-resistant mutant of *A. brasilense* Sp13t, isolated from *Digitaria decumbens* Stent (L.). The mutant strain used in the work reported here, resistant to both

streptomycin and rifampin, was developed by A. Wood and D. E. Duggan, Department of Microbiology and Cell Science, University of Florida. The substrate-buffered medium described by Tyler et al. (M. E. Tyler, D. A. Zuberer, and J. R. Milam, Abstr. Proc. Steenbock-Kettering Int. Symp. N_2 Fixation, 1978), containing equimolar quantities of succinate and fructose, was used to produce bacterial cultures. Yeast extract, Trypticase, and biotin were omitted to avoid misinterpretation of phytohormone assay results. The bacteria were grown in 500-ml flasks containing 200 ml of the N-free medium, amended with 100 μg of DL-tryptophan per ml at 28°C in the dark unless otherwise specified. The bacterial population was determined by measuring the light absorption at 670 nm and by standard plate count techniques. The agar medium used in the plate count contained Döbereiner N-free medium (8), amended with 100 mg of streptomycin, 100 mg of rifampin, 150 mg of cycloheximide, 100 mg of safranin, and 50 mg of vitamin-free Casamino Acids in 1 liter.

Extraction process. Bacterial cultures (200 ml) were centrifuged at 7,700 × *g* for 30 min. The supernatant was reduced to 50 ml by evaporation under vacuum and extracted into ethyl acetate and *n*-butanol fractions by the procedure outlined in Fig. 1. The extracts were filtered through 0.45-μm membrane filters, and chromatographed by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC).

Identification. Cochromatography with authentic compounds by TLC, specific color reactions with chromogenic reagents, HPLC, and bioassays were used to establish identity. TLC chromatograms were run on 0.50-mm-thick preparative silica gel plates. Solvent systems were chloroform:ethyl acetate:formic acid (50:40:10 [vol/vol]) to separate indole compounds and gibberellins in ethyl acetate fractions, and *n*-butanol:acetic acid:water (12:3:5 [vol/vol]) to separate cytokinins in *n*-butanol fractions. IAA and other indole compounds were detected on TLC plates by spraying with Ehrlich reagent (2). Gibberellins were detected by spraying the chromatograms with ethanolic sulfuric acid (90:10 [vol/vol]) and heating to induce fluorescence of the compounds in ultraviolet light (19).

HPLC chromatograms were produced by injecting 5 to 10 μl of the filtered extracts onto a 10-μm reverse-phase column (Waters Associates μBondapak C_{18}, 4 mm by 30 cm) in a Waters Associates liquid chromatograph equipped with a differential ultraviolet detector absorbing at 254 nm. Two solvent systems were used to separate indole compounds and cytokinins. Solvent A was water:acetonitrile:acetic acid (85:15:1 [vol/vol]), flow rate was 1.5 ml/min, and the operating pressure was 1,400 lb/in^2 (95 atm). Solvent B was 30% methanol in water, flow rate was 1.5 ml/min, and the operating pressure was 1,600 lb/in^2 (108 atm). Retention times for peaks were compared to those of authentic standards added to the medium and extracted by the same procedures used with bacterial cultures. Quantitation was by comparison of peak heights. Separations of some indole compounds and cytokinins by HPLC are shown in Fig. 2 and 3.

For bioassay procedures, TLC chromatograms not treated with chromogenic reagents were dried and cut transversely into 10 equal sections representing the sequence of R_f values 0.1 to 1.0. These sections were then eluted separately for bioassays. The spot on the

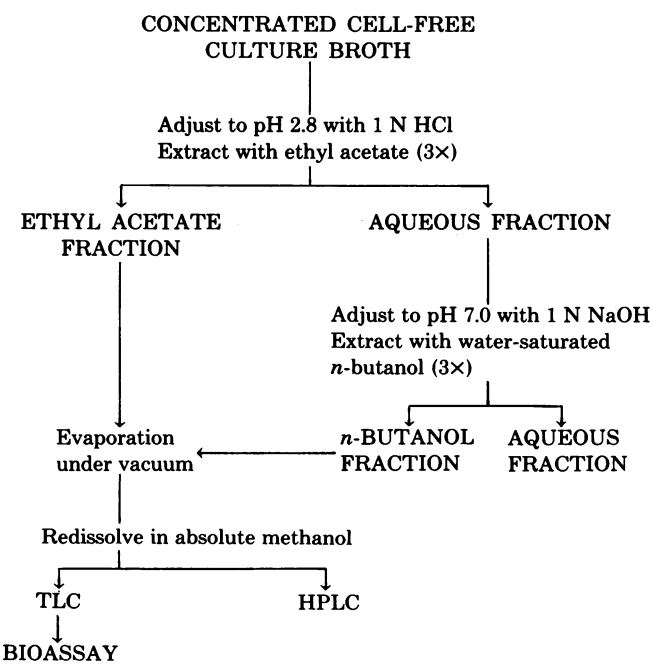

FIG. 1. *Procedures used in extracting and partitioning bacterial cultures.*

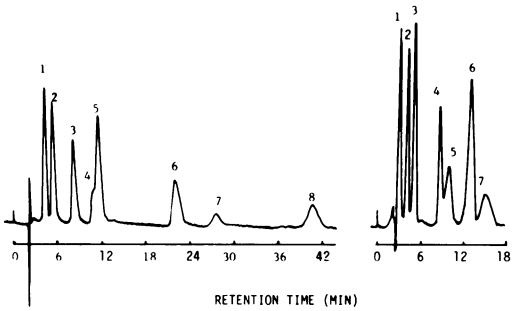

FIG. 2. *HPLC separation of a mixture of authentic indoles by the two solvent systems described in text. Mixtures of indoles contained 25 μg/ml each, and injection volume was 10 μl. Solvent mixture A (left): 1, TTP; 2, tryptamine; 3, indole-3-lactic acid; 4, indole-3-acetaldehyde; 5, IAA; 6, indole propionic acid; 7, indole pyruvic acid; 8, indole butyric acid. Solvent mixture B (right): 1, ILA; 2, tryptamine; 3, IAA; 4, indole-pyruvic acid; 5, indole-3-acetaldehyde; 6, indole acetronitrile; 7, indole-butyric acid.*

plate at the R_f of IAA was extracted with methanol and tested for effects on oat coleoptile segment elongation at 25°C by the method of Bentley (2). Gibberellin-like substances were assayed by the method of Frankland and Wareing (10) with lettuce hypocotyls. Cytokinins were detected by effects of extracts on chlorophyll retention in oat leaves. The method described by Strain et al. (31) was used to determine chlorophyll content.

Plant response to inoculation and plant growth hormone treatments. Seeds of pearl millet [*Pennisetum americanum* (L.) Shum cv. Gahi 3] were surface sterilized with 95% ethanol and 2.5% sodium hypochlorite. Seedlings were grown on a stainless steel mesh support inserted into tubes (25 by 3 cm) containing 150 ml of dilute Hoagland mineral nutrient solution (¼ strength). Tubes were covered with inverted 50-ml beakers. At 2 days after germination, 2 ml of bacterial culture (5×10^7 cells per ml) was added to the nutrient solutions. Lateral root and root hair formation was examined, and the length of roots, the number of lateral roots, and the fresh weights of tops and roots were recorded after 30 days.

The effect of purified plant hormones on the growth of plants was studied by growing seedlings as described above, except that plants were grown in open tubes with aerated nutrient solutions. Plant hormones were added to the nutrient solutions at planting and 10 days after planting. Plants were grown in a greenhouse for 3 weeks before recording plant growth data.

RESULTS AND DISCUSSION

IAA. The production of IAA from tryptophan (TPP) by *Azospirillum brasilense* Sp13t SR2 was confirmed by TLC, HPLC, and bioassay. TLC of ethyl acetate extracts from *A. brasilense* cultures always showed a clear blue spot at the R_f corresponding to authentic IAA, when chromatograms were treated with Ehrlich reagent. Figure 4 shows responses of oat coleoptiles to different concentrations of the "IAA fraction" from a 15-day-old culture. A HPLC chromatogram of this fraction is shown in Fig. 5a. The peak at 13.8 min of retention time was not seen in the chromatogram of the whole ethyl acetate fraction (Fig. 5b). This suggests that the sub-

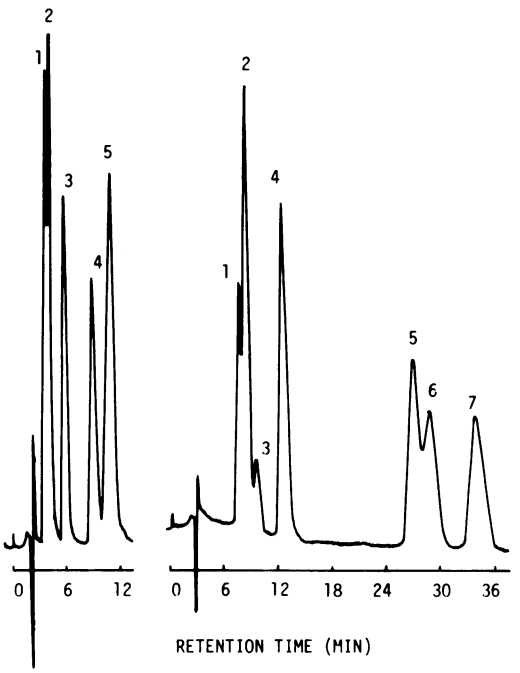

FIG. 3. *HPLC separation of a mixture of authentic cytokinins by the two solvent systems described in the text. Mixtures contained 15 μg/ml each, and injection volume was 10 μl. Solvent mixture A (left): 1, zeatin; 2, zeatin riboside; 3, kinetin; 4, N-6(Δ-2-isopentenyl)adenine; 5, N-6(Δ-isopentenyl)adenine riboside. Solvent mixture B (right): 1, zeatin riboside; 2, zeatin trans/trans; 3, zeatin cis/trans; 4, kinetin; 5, benzyladenine; ;6, N-6(Δ-isopentenyl)adenine riboside; 7, N-6(Δ-isopentenyl)adenine.*

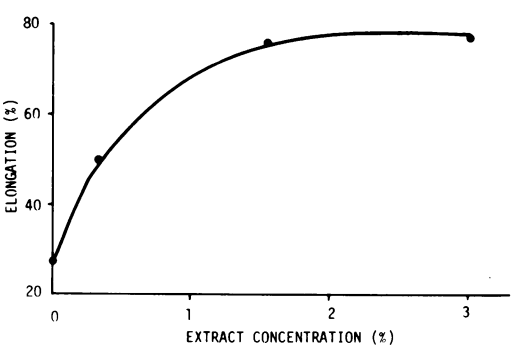

FIG. 4. *Avena sativa coleoptile response to different concentrations of IAA fraction from ethyl acetate extract of A. brasilense Sp13t SR2 culture solution.*

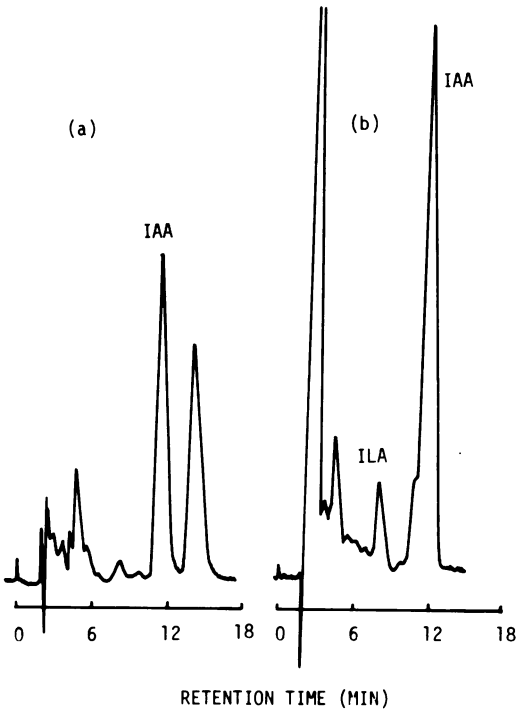

FIG. 5. *HPLC separation of (a) the IAA fraction of an A. brasilense Sp13t SR2 culture solution after TLC separation, and (B) an ethyl acetate extract of the same fraction.*

stance may be an IAA degradation product. The solvent system used in TLC to separate indole compounds has been shown to cause some destruction of IAA (27). TLC plates showed a second blue spot at the R_f of indole-3-lactic acid (ILA). The methanol extract of this spot did not cause elongation of oat coleoptiles. HPLC indicated that this compound was ILA (Fig. 5b). Perley and Stowe (23) suggested that ILA is interconverted to indole pyruvic acid, a precursor of IAA in higher plants, in cultures of *Bacillus cereus*. Indole pyruvic acid is notoriously unstable (28) and therefore difficult to detect reliably.

Effect of tryptophan concentration in medium. IAA production increased with increasing concentration of TPP from 1 to 100 μg/ml in culture medium (Fig. 6). Although autoclaving may induce formation of IAA from TPP, none was detected by HPLC in sterile, autoclaved media containing less than 100 μg of TPP per ml. At higher concentrations, some IAA could be detected. Data in Fig. 6 show IAA production by 5-day-old *A. brasilense* cultures growing in N-free medium containing different concentrations of DL-tryptophan. Bacterial growth, which was very slow in medium lacking TPP, increased markedly when 1 μg of TPP was added to the N-free medium. Higher concentrations retarded growth, however, and growth stopped when the concentration reached 1 mg/ml (data not shown). This toxicity may be due to D-TPP (7). The increased growth rate resulting from addition of TPP was probably due to utilization of TPP by the bacteria as a sole nitrogen source (7).

Effect of culture ages. The production of IAA and ILA by *A. brasilense* growing in stationary culture and N-free medium at different ages is shown in Fig. 7. IAA production was proportional to the bacterial population in the medium, when other factors were not limiting. ILA production also increased with time but much less rapidly than IAA.

Effect of culture and medium conditions. Studies of the influence of the medium and of culture conditions were made with 4-day-old cultures (Table 1). Shaking greatly increased growth and IAA production by bacteria in medium containing ammonium chloride (1 g of

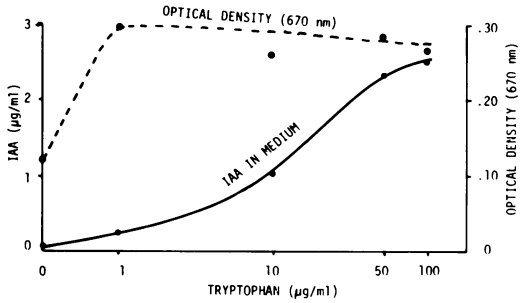

FIG. 6. *Effects of tryptophan concentration in medium on the growth and IAA production of A. brasilense Sp13t SR2.*

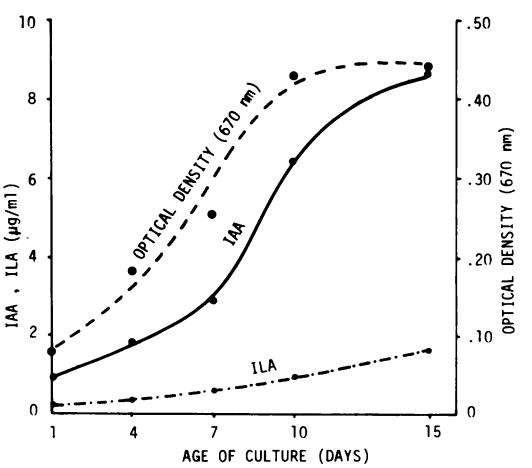

FIG. 7. *Time course of the production of IAA and ILA by Azospirillum brasilense Sp13t SR2.*

TABLE 1. *Effect of medium and culture conditions on the production of IAA by A. brasilense Sp13t SR2*

Conditions	A_{670}[a]	IAA (μg/ml)
N-free medium		
Stationary	0.18	1.9
Shaking	0.16	2.0
NH_4 medium		
Stationary	0.85	2.6
Shaking	1.10	24.4

[a] A_{670}, Absorbance at 670 nm.

NH_4Cl per liter). The increase in IAA was about 10-fold in shake cultures after 4 days. In N-free medium growth was slower, and IAA production was only slightly increased by shaking. The results suggest that fixed nitrogen had little effect on the production of IAA by *A. brasilense*. The effects of shaking, bacterial population, and TPP concentration were more important. Lee et al. (17) reported that the IAA production of *Azotobacter vinelandii* was reduced in the presence of fixed nitrogen in both shaking and stationary conditions, but did not mention the bacterial population. The slight decrease of bacterial growth in N-free medium with shaking may have resulted from suppression of nitrogenase activity by the increased oxygen concentration that resulted from the shaking.

Gibberellin-like substances. Ethyl acetate extracts were prepared from cultures of *A. brasilense* grown under stationary conditions in N-free medium for 7 days. Because gibberellic acids (GAs) do not absorb ultraviolet light at 254 nm, only TLC and bioassays were used for their detection. TLC showed unidentified substances which fluoresced green under ultraviolet light at R_f 0.1 and 0.2, and others which fluoresced pale yellow at R_f 0.4 to 0.6. The pale yellow fluorescence may be emitted by the interfering substances. The R_f value of GA_3 in this solvent system is 0.5 to 0.6, and it fluoresces green.

The substances in the ethyl acetate fraction of *A. brasilense* at R_f 0.6 significantly increased growth of lettuce hypocotyls (Fig. 8). Response to other fractions was not significantly different from that of control. Calculated from a response curve developed with pure GA_3, *A. brasilense* produced about 0.05 μg of GA_3 equivalent per ml in the culture medium. A mixture of several gibberellins may be present, but these could not be separated with the solvents used.

Cytokinin-like substances. The *n*-butanol fraction of a 10-day-old *A. brasilense* culture in N-free medium was used for detection of cytokinins. Most cytokinins, especially Z and ZR, are retained in this fraction (13, 18). The effect of *n*-butanol fractions on chlorophyll retention is shown in Fig. 9. Three response areas are shown clearly at R_f 0.2 to 0.3, 0.5 to 0.7, and 0.8 to 1.0. In this solvent system, the R_f of both zeatin and zeatin riboside is 0.6. We cannot identify this cytokinin on HPLC chromatograms because of the presence of interfering substances. Further purification is needed before analyzing the *n*-butanol fraction by HPLC. Bioassay of the whole *n*-butanol fraction (without separation by TLC) showed a very high chlorophyll retention (up to 0.7 mg/g of fresh weight of oat leaves, equivalent to about 0.001 μg of kinetin per ml).

Response of plants. Roots of pearl millet seedlings showed a striking growth response when the root solutions were inoculated with *A. brasilense* (Table 2). Almost all lateral roots were densely covered by root hairs (Fig. 10A), whereas very few or none developed on uninoculated control plants (Fig. 10B). A few weeks after inoculation, the root solutions were clear, and bacterial cells were rarely found when portions of the undisturbed solutions were exam-

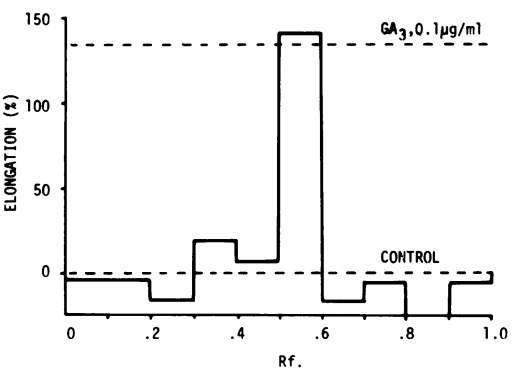

FIG. 8. *Effect of ethyl acetate fraction of A. brasilense Sp13t SR2 culture on elongation of lettuce (Lactuca sativa) hypocotyls.*

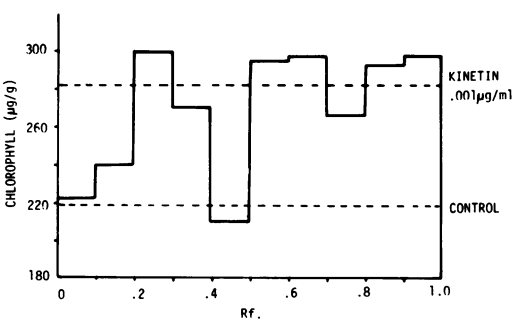

FIG. 9. *Effect of n-butanol fraction of A. brasilense Sp13t SR2 culture on chlorophyll retention by oat (Avena sativa) leaves.*

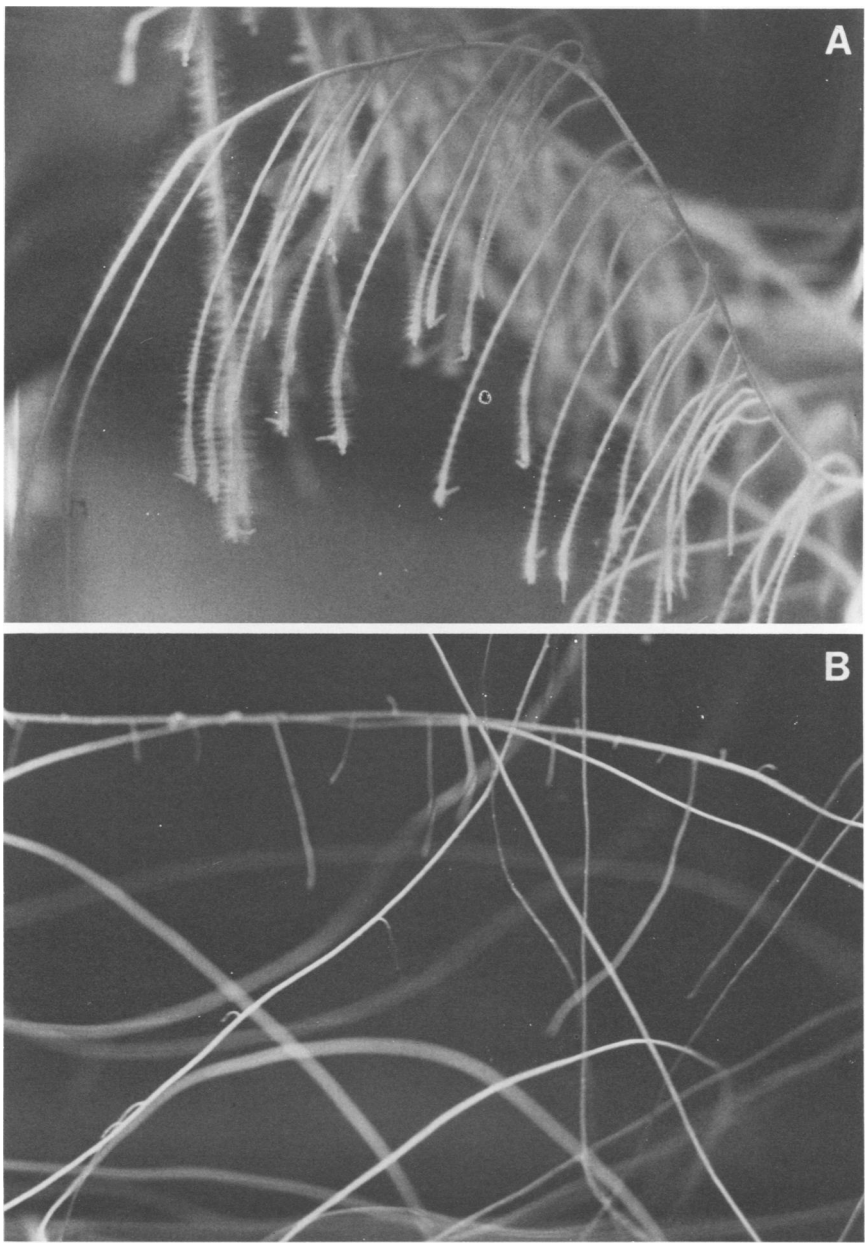

FIG. 10. *Response of the root systems of pearl millet to (A) inoculation of Azospirillum brasilense Sp13t SR2, (B) uninoculated control, (C) authentic plant hormones (IAA, GA, kinetin). Photos show plants at week 6.*

ined by light microscopy. However, the root hairs were densely covered with bacterial cells, even at 1 or 2 months after inoculation. These observations confirm those reported in detail by Garcia et al. (11; M. U. Garcia, D. H. Hubbell, M. H. Gaskins, and F. B. Dazzo, Abstr. Proc. Steenbock-Kettering Int. Symp. N_2-Fixation, 1978). Pecket (21) reported that GA enhanced the formation of root hairs on excised pea roots. Torrey (33) summarized evidence that GA appears to play no role in lateral root initiation. But in our experiment, GA_3 increased the number of lateral roots of pearl millet at a concentration as low as 0.005 μg/ml (1.4×10^{-8} M). No

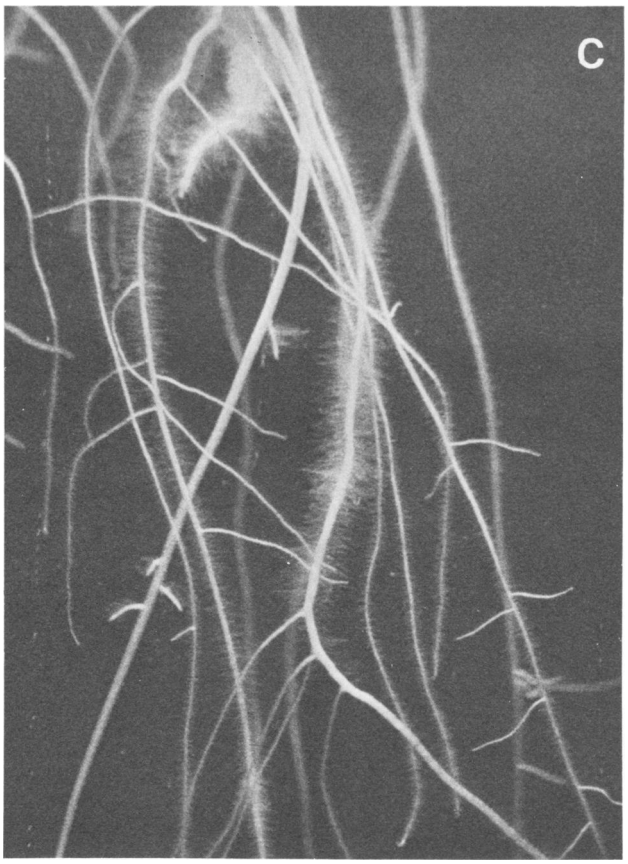

FIG. 10 C

effect on root hair production was observed at any rates of GA_3. This effect of GA_3 on pearl millet roots is similar to that on excised tomato roots (6).

Cytokinin (as kinetin) at levels of 0.005 μg/ml (1.4×10^{-8} M) and above reduced main root length and lateral root development. Although cytokinin is known to have a role in the control of lateral root initiation (32), no reports have been found concerning cytokinin effects on root hair formation. In our experiment, the density of root hairs was proportional to the level of kinetin in the solution to a concentration of 0.01 μg/ml (2.7×10^{-8} M). At this point, both main and lateral roots were covered with root hairs. Root weights were little affected by kinetin, but top weights were significantly increased by a concentration of 0.001 μg of kinetin per ml (Table 3).

IAA at the concentrations tested had little effect on lateral root production but 0.01 μg/ml (6×10^{-8} M) significantly increased top weight (Table 3). The root system is more sensitive to auxin than the shoot. Thimann (32) reported that initiation of lateral roots on the main root is certainly promoted by auxin. He showed that the elongation of oat seedling roots was inhibited, but the number of roots formed was greatly increased when oat seeds were germinated in auxin solution.

A combination of IAA, GA_3, and kinetin increased plant growth when the concentration of IAA and kinetin were very low (0.005 and 0.001 μg/ml, respectively). Higher concentrations of IAA and kinetin appeared to reduce growth, although differences observed were not statistically significant. Root hairs and lateral roots were more dense when IAA, GA_3, and kinetin were added to the solution. These effects on morphology of pearl millet roots were similar to those produced by inoculation with *A. brasilense*. However, root hairs on inoculated plants were more dense and longer than on plants grown with pure hormones (Fig. 10C).

We do not find it surprising that some differences are found between plant response to pure

TABLE 2. *Effect of A. brasilense Sp13t SR2 inoculation on the root system of pearl millet grown in N-free dilute Hoagland solution*[a]

Plant type	Fresh wt of top[b] (mg/plant)	Fresh wt of root[b] (mg/plant)	No. of lateral roots[b]	Main root length[c] (cm)	Total lateral root length[b] (cm)
Inoculated	74.47 (4.31)	12.05 (0.57)	56 (9)	40.40 (2.96)	48.70 (10.04)
Uninoculated	52.67 (2.46)	12.01 (0.87)	38 (4)	50.30 (3.14)	39.40 (4.57)

[a] Plants were grown in closed tubes (25 by 3 cm) containing 150 ml of N-free, diluted Hoagland solution. Bacteria were added to the inoculated tubes 2 days after planting, and plants were harvested 30 days thereafter.
[b] Means are for 10 plants. Figures in parentheses indicate standard error of the mean.

TABLE 3. *Effect of plant growth hormones and inoculation with A. brasilense Sp13t SR2 on the growth of pearl millet*[a]

Categories	Concn (μg/ml)	Root fresh wt (mg)	Top fresh wt (mg)
Control		161 a[b]	694 b
IAA	0.05	189 a	866 ab
	0.01	200 a	990 a
	0.005	173 a	838 ab
GA_3	0.05	168 a	832 ab
	0.01	140 a	756 ab
	0.005	152 a	829 ab
Kinetin	0.01	176 a	629 b
	0.005	152 a	638 b
	0.001	181 a	923 a
IAA + GA_3 + kinetin	0.01 + 0.05 + 0.001	155 a	822 ab
	0.01 + 0.05 + 0.005	137 a	694 b
	0.005 + 0.05 + 0.001	181 a	892 a
Inoculated		211 a	1017 a

[a] Plants were grown in tubes (25 by 3 cm) containing 150 ml of diluted Hoagland solution with 21 mg of N per liter. Plant hormones were added to the solutions at planting and 10 days after planting. Bacteria were added to the inoculated treatments 2 days after planting. All plants were grown in open air in the greenhouse for 3 weeks.
[b] Values within columns followed by the same letter are not significantly different at the 0.05 level of probability.

plant growth substances and plant response to bacterial inoculation. Substances produced by bacteria are released continuously, and, especially in the case of *A. brasilense,* they are produced on the surfaces or within the plant tissue since the bacteria grow there (Garcia et al., Abstr. Proc. Steenbock-Kettering Int. Symp. N_2-Fixation). It seems probable that plant growth substances produced by *A. brasilense* improve plant growth by their direct effects on metabolic processes. However, since they induce proliferation of lateral roots and root hairs and thus increase nutrient absorbing surfaces, this may lead to greater rates of nutrient absorption. This in turn would be expected to increase plant growth. In both N-free closed systems and open systems, *A. brasilense* significantly increased the dry weight of the plants. Acetylene reduction assays of plants grown in N-free Hoagland solution were negative for both inoculated and uninoculated treatments. Thus, the plant growth response could not be attributed to dinitrogen fixation by the bacteria. Although this organism achieves high fixation rates under microaerophilic conditions (8) attempts to induce such activity in the rhizosphere have met with limited success (Gaskins and Hubbell, in press). Thus, data are not yet available to indicate the relative importance of dinitrogen fixation and growth hormone production in the enhancement of plant growth by *Azospirillum* under field conditions.

ACKNOWLEDGMENTS

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